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Engineered sodium hyaluronate respirable dry powders for pulmonary drug delivery

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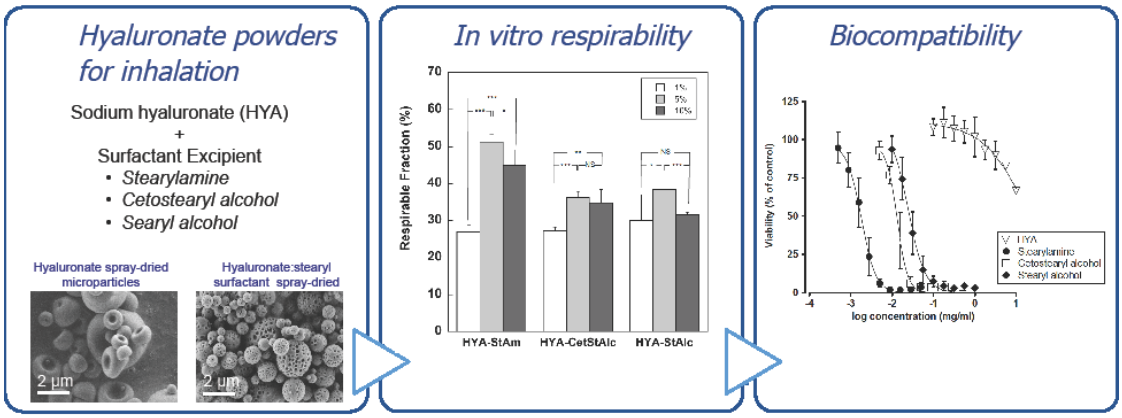
Graphical abstract



Abstract:

Sodium hyaluronate (HYA) warrants attention as a material for inhalation due to its (i) therapeutic potential, (ii) utility as a formulation excipient or drug carrier, and (iii) ability to target lung inflammation and cancer. This study aimed to overcome formulation and manufacturing impediments to engineer biocompatible spray-dried HYA powders for inhalation.

Novel methodology was developed to produce HYA microparticles by spray drying. Different types of surfactant were included in the formulation to improve powder respirability, which was evaluated *in vitro* using cascade impactors. The individual formulation components and formulated products were evaluated for their biocompatibility with A549 respiratory epithelial cells.

The inclusion of stearyl surfactants, 5% w/v, produced the most respirable HYA-powders; FPF 59.0-66.3%. A trend to marginally higher respirability was observed for powders containing stearylamine > stearyl alcohol > cetostearyl alcohol. Pure HYA was biocompatible with A549 cells at all concentrations measured, but the biocompatibility of the stearyl surfactants (based on lethal concentration 50%; LC_{50}) in the MTT assay ranked stearyl alcohol > cetostearyl alcohol > stearylamine with LC_{50} of 24.7, 13.2 and 1.8 μg/mL, respectively.

We report the first respirable HYA powders produced by spray-drying. A lead formulation containing 5% stearyl alcohol was identified for further studies aimed at translating the proposed benefits of inhaled HYA into safe and clinically effective HYA products.

Key words: Hyaluronic acid, hyaluronan, inhalation toxicology, dry powder inhaler, particle engineering

1. Introduction

Sodium hyaluronate (HYA) warrants attention as a product for inhalation on account of (i) having inherent therapeutic potential, (ii) offering utility as a formulation excipient or drug carrier, and (iii) possessing targeting potential for disease sites in the lungs, e.g. macrophages or lung cancer (Liao et al., 2008). The development of inhaled formulations has been limited by difficulty in formulating and manufacturing sodium hyaluronate as a powder for inhalation, therefore this study aimed to engineer a flowable, highly respirable powder.

Hyaluronate is an endogenous glycosaminoglycan present in matrices such as the extracellular matrix and synovial fluid. It is a linear polysaccharide composed of a repeating disaccharide unit of N-acetyl-D-glucosamine and D-glucuronic acid bound by β 1,4 glycosidic bond. The disaccharides are linked by β 1,3 bonds to form the hyaluronic acid chains (Lapcík L Jr and et al., 1998). *In vivo*, hyaluronate exists as a polyanion rather than its protonated form, hyaluronic acid, although the terms are often used synonymously in the literature (Liao et al., 2008). The use of high molecular weight sodium hyaluronate is approved in several pharmaceutical products for topical application and injection and as a functional component of some nebuliser solutions. Hence, hyaluronate is considered to be a biocompatible, biodegradable and non-immunogenic biomaterial.

In the lungs, the physiological function of hyaluronate is to stabilize connective tissue, organize extracellular matrix, control hydration / water homeostasis (Gerdin and Hällgren, 1997), as well as modulating cell migration and phagocytosis (Turino and Cantor, 2003). In disease hyaluronate plays a role in the inflammatory response (Cantor, 2007), tissue remodeling (Petrigni and Allegra, 2006) and various CD44 receptor-mediated functions in cell detachment, carcinogenesis and inflammation (Zhong et al., 2016). The CD44 receptor belongs to the family of cell adhesion molecules specifically involved in the control of cell behaviour by mediating contact between cells or between cells and the extracellular matrix, which is essential for maintaining tissue integrity (Arpicco et al., 2013). However, these important functions are also central to pathological conditions including tumour progression and metastasis (Orian-Rousseau, 2010). CD44 receptors bind high molecular weight hyaluronate, but can also interact with shorter chains (Tammi et al., 2002).

Several studies have reported hyaluronate to be a potential therapeutic agent for inflammatory lung disorders, in particular for prevention of exercise-induced bronchoconstriction in asthma, emphysema and COPD (Petrigni and Allegra, 2006; Souza-Fernandes et al., 2006). Different

mechanisms of actions linked to the inhibition of lung elastase, binding to elastic fibers and limitation of cell-cytokine interactions via CD44 receptor have been described (Cantor et al., 2011; Iskandar et al., 2009). HYA is an active component of two inhalation products. Yabro® (Ibsa Farmaceutici, IT) is a high viscosity hyaluronate solution for nebulization (MW 800-1000 kDa, 0.3% w/v) to reduce bronchial reactivity induced by inhalation of allergens/pollutants or by physical effort (Gelardi et al., 2013). In this formulation, HYA improves palatability and reduces potential side effects of nebuliser solutions such as irritation and cough. Hyaluronate (MW 500 kDa, 0.1 % w/v) is also available in a hypertonic solution of NaCl at 7% (Hyaneb®, Chiesi Farmaceutici, IT) which decreases mucus viscosity in cystic fibrosis patients by attracting water to hydrate the mucus (Nenna et al., 2011). Hyaluronate has also been proposed as carrier for drug delivery to the lung, either as a scaffold for modified release formulations or for particle/drug targeting to alveolar macrophages, e.g. for treatment of tuberculosis (Hwang et al., 2008).

Despite this potential, HYA has not been developed as a powder formulation for lung delivery, either alone or in combination with drugs. HYA particles have poor flowability and tend to be cohesive requiring the use an adhesive mixture with lactose in order to be aerosolized (Hwang et al., 2008). The aim of the present work was to investigate the use of different excipients to produce flowable, highly respirable and safe hyaluronate dry powders through a particle engineering approach based on spray drying. Powder respirability was investigated *in vitro* and prototype formulations were evaluated for alveolar cell compatibility *in vitro*.

2 Materials and methods

2.1 Materials

Sodium hyaluronate (HYA; PrymalHyal 50, average MW=29504 Da) was purchased from Soliance (Pomacle, France). Stearylamine, L-lysine and thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), N,N-Dimethylformamide (DMF), RPMI-1640, Fetal Bovine Serum (FBS), L-glutamine, gentamicin were supplied by Sigma–Aldrich (Sigma Chemical Co., Milan, Italy). Stearyl alcohol, cetostearyl alcohol and stearylamine were purchased from ACEF Srl (Fiorenzuola d'Arda, IT). A single dose dry powder inhaler, RS01 (Plastiape Spa, LC, IT), was used to aerosolize HYA powders for aerodynamic performance testing. Powder formulations were loaded in size 3 hypromellose capsules (Qualicaps Europe, Madrid, ES). All chemicals used were of analytical grade and water was purified (0.055 µS/cm, TOC 1ppb) with Purelab pulse + Flex ultra-pure water (Elga Veolia, Milan, IT). A549 alveolar epithelial cells were obtained from the American Type Cell Culture; tissue culture flasks (75 cm^2 with ventilated caps) and 96-well plates were from Costar (Fisher Scientific, Loughborough, UK). Phosphate buffered saline (PBS) tablets were purchased from Oxoid Ltd (Basingstoke, UK).

2.2 HPLC analysis of sodium hyaluronate (HYA)

The content of HYA in every sample was determined by size exclusion – high performance liquid chromatography (SEC-HPLC) using a BioSep-SEC-s4000, 5 µm 7.8x100 mm column (Phenomenex Srl, Bologna, IT). Standard and samples were prepared in purified water. Mobile phase was prepared by dissolving 6.80 g of KH_2PO_4 in 1 L of purified water and the pH was adjusted to 7.0 with 5 M potassium hydroxide. The injection volume was set at 100 µL, flow rate of the mobile phase was 1.0 mL/min and wavelength of detection was 200 nm. Linearity of response was tested before each analysis in the concentration range between 5 and 500 µg/mL ($R^2 = 0.999$).

2.3 Production of HYA powders by spray drying

HYA powders were manufactured by spray-drying using a mini spray-dryer B-290 (BÜCHI Labortechnik AG, Flawil, Switzerland). HYA was dissolved in purified water at room temperature and this solution was added to ethanol (water:ethanol ratio 30:70 v/v) under magnetic stirring at 50 rpm. When excipients were incorporated in the formulation, they were added to the water or ethanol phase (according to excipient solubility). The compositions of hyaluronate formulations are reported in Tables 1 and 2.

The solutions were spray-dried using the following process parameters: inlet temperature 90 °C, drying air flow rate 750 L/h, solution feed rate of 3.0 mL/min and nozzle diameter of 0.7 mm. Under these conditions an outlet temperature ranging from 45 to 52 °C was measured. A dehumidifier B-296 was used to control the air humidity of the system. Spray-dried powders were kept in the collector for at least 24 hours before use in order to reduce electrostatic charges.

2.4 Particle size distribution

The particle size distribution of the powders was measured using a laser light diffractometer Spraytec (Malvern Instruments Ltd, Worcestershire, UK) equipped with a 300 mm focal lens, which measures particle size in the range 0.1 to 900 μm. Samples were prepared by suspending 10 mg of the spray-dried powder in 10 mL of a solution of Span 85 (0.1 % w/v) in cyclohexane; the suspension was sonicated for 10 min to achieve complete particle dispersion. Particle size distribution was measured in triplicate with an obscuration threshold of 8%. Data were expressed as volume diameter of 10^{th} (Dv10), 50^{th} (Dv50) and 90^{th} (Dv90) percentile of the particle population and as span value [(Dv90-Dv10)/Dv50].

2.5 Scanning Electron Microscopy

Scanning electron microscopy (SUPRA 40, Carl Zeiss NTS GmbH, Oberkochen, Germany) was employed to investigate particle morphology and surface characteristics of the powders produced by spray-drying. The microscope was operated under high vacuum conditions with

1.5 kV accelerating voltage, at different magnifications. Powders were deposited on adhesive black carbon tabs pre-mounted on aluminium stubs and imaged without any metallization process.

2.6 Thermo gravimetric Analysis (TGA)

Thermogravimetric analysis (TGA/DSC1 STAR^e System, Mettler Toledo, USA) was carried out on powder samples placed in 70 μL alumina pans with a pierced cover. Samples were heated under a flux of dry nitrogen (100 mL min^{-1}) at 10 K min^{-1} in the 30-150 °C temperature range.

2.7 Differential Scanning Calorimetry (DSC)

DSC measurements were performed on an Indium calibrated (onset of melting T_m = 157.1 °C, enthalpy of melting ΔH_m = -27.84 J g^{-1}) Mettler DSC 821e (Mettler Toledo, USA) driven by STARe software (Mettler Toledo). DSC traces were recorded by placing precisely weighed quantities (6-9 mg) in a sealed and pierced 40 μL aluminium pan. Scans were performed between 25 to 150 °C at 10 K min^{-1} under a flux of dry nitrogen (100 mL min^{-1}). Each powder sample was analysed at least in duplicate. Data relevant to the observed thermal events were reported as peak temperatures

2.8 Aerodynamic performance

A fast screening impactor (FSI, Copley Scientific Ltd, Nottingham, UK) was used as an abbreviated impactor to assess the aerodynamic performance of HYA powders. FSI is constituted of a Coarse Fraction Collector (CFC) that captures particles with an aerodynamic diameter larger than 5 μm and a Fine Fraction Collector (FFC) that collects particles with an aerodynamic diameter smaller than 5 μm. The respirable fraction was calculated as the ratio between the amount of powder collected in the FFC and the loaded dose. The distribution of hyaluronate particles in the FSI was quantified by HPLC. The entire system was connected to

a vacuum pump mod. 1000 (Erweka GmbH, Germany) which created the air flow rate to aerosolise the powder and distribute it in the FSI.

HYA powders (5 mg) were loaded manually into a size 3 hypromellose capsule and aerosolized using a RS01 powder inhaler device. A single capsule was discharged inside the impactor for each test. The flow rate used during each test was adjusted, according to current USP monograph, with a Critical Flow Controller TPK (Copley Scientific, Nottingham, UK) in order to produce a pressure drop of 4 kPa over the inhaler. Thus, a flow rate of 60 L/min was set before each experiment by a Flow Meter DFM 2000 (Copley Scientific, Nottingham, UK). RS01 was activated and the vacuum applied for 4 seconds so that a volume of 4 L of air was drawn through the inhaler during the experiment.

Andersen cascade impactor (ACI, Copley Scientific Ltd, Nottingham, UK) was employed in order to characterize in greater detail the aerodynamic behavior of the stearyl- surfactant formulations. The ACI was assembled for use at flow rate of 60 L/min. Plates of each stage of the impactor were coated with a 1% w/v Span 85 solution in cyclohexane to prevent particles bouncing during the particle deposition. A dose of 5 mg of powder was loaded in a hypromellose capsule and aerosolized using an RS01 device as described above. *In vitro* deposition experiments were performed in triplicate.

The measurement of the HYA deposited in the impactor allowed the calculation of deposition parameters: the delivered dose (DD) was the amount of HYA ex-device measured from the induction port (IP) to the filter (F). The fine particle dose (FPD) was the mass of HYA with aerodynamic diameter lower than 5 μm; the fine particle fraction (FPF) was the ratio between FPD and DD. The mass median aerodynamic diameter (MMAD) was determined by plotting the cumulative percentage of mass less than the stated aerodynamic diameter (probability scale) versus aerodynamic diameter (log scale).

2.9 Biocompatibility with A549 alveolar epithelial cells

The A549 cell lines were obtained from the American Type Cell Culture and used between passages 90-110 to perform all biocompatibility experiments. Cells were grown in 75 cm^2 flasks (Costar Corning, UK) in a humidified 5% CO_2/95% atmospheric air incubator at 37 °C. RPMI-1640 medium supplemented with 10% v/v foetal bovine serum (FBS), 1% v/v L-glutamine and 0.1% v/v gentamycin was used as cell culture medium (CCM).

Cell viability was measured by a reduction in metabolic activity measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the MTT assay, cells were detached using trypsin-EDTA 0.25%:0.02% once they reached 80-85% of confluence and seeded in 96-well plates at a density of 10,000 cells per well (in 200 µL CCM). The cells were incubated for 24 h to allow the cells to attach and form a monolayer. Prior to cytotoxicity test, the CCM was removed, replaced with 100 µL of fresh CCM containing the test concentrations of the stearyl surfactants, individually and in the formulations with HYA.

Biocompatibility of the individual components (HYA, stearylamine, cetostearyl and stearyl alcohol) and the spray dried powders (HYA:surfactant 90:10) was evaluated over 24 h. The test powders were dissolved in a medium:ethanol mixture (95:5) and incubated at 37°C for 1 h before addition to the cells. All materials were tested over 9 different concentrations following a serial quarter log dilution and a CCM:ethanol (95:5) mixture was used as a negative control.

After 24 h, cells were washed with PBS and 200 µL of fresh CCM was added. Finally, 50 µL of MTT solution (2.5 mg/mL in PBS) were added to each well and the plate was incubated for 4 h in a humidified incubator, after which the solution was discarded. The cells were lysed and formazan crystals formed were solubilised with 100 µL of a solution of 10% SDS in DMF:water (1:1). Cells were incubated with lysis solution overnight at 37°C before the

absorbance of solubilised formazan was measured at 570 nm using a SpectraMax microplate reader (Molecular Devices, UK). The cell viability was expressed as a percentage of negative control (100% metabolic activity). Lethal concentration 50% (LC_{50}) values were calculated as the concentration that caused 50% reduction in MTT conversion from the sigmoidal relationship obtained by plotting $\log_{10}$ concentration of surfactant vs % cellular viability using GraphPad Prism (GraphPad Software, US).

2.10 Statistical analysis

The statistical analysis was performed with Microsoft Office Excel 15.1 (Microsoft Corp.) employing a two-tailed unpaired t-test with significance level fixed at p-value = 0.05.

3. Results

3.1 Spray dried sodium hyaluronate powders with respirable attributes

Hyaluronate powders from aqueous solutions of sodium hyaluronate could not be produced by spray drying since powder formed in the cyclone of the spray drier and the yield was only around 10%. In order to accelerate the particle formation during the spray drying process, ethanol (70% v/v) was added to the aqueous solution of sodium hyaluronate. In this way, an adequate yield (68% by weight) was obtained, starting from solutions with 0.83-0.92% hyaluronate content (Table 1). The hyaluronate content of the powder was around 90%, and water content determined by TGA around 10%, thus affording a correct mass balance. The particle size distribution showed that this powder had a promising geometrical size (d_{v50} 2.09) and a narrow distribution around the median value as indicated by a span of 0.45 (Figure 1A). However, the spray-dried hyaluronate powder was cohesive and characterised by the presence of many visible agglomerates. When aerosolized using RS01 device into a fast screening impactor, 50% of the dose was retained inside the capsule, the capsule chamber and mouthpiece, indicating poor flowability (see Figure 1B) as a consequence of HYA microparticle cohesion. The emitted dose did not de-aggregate completely leading to a low respirable fraction of 25%.

It was hypothesized that the high cohesiveness was due to the strong negative surface charge of the sodium hyaluronate. It is known that electrostatic charge is one of five mechanisms that govern the deposition of inhaled particles in the lungs (Karner and Urbanetz, 2011; Wong et al., 2015). Furthermore, the electrostatic charge of the plastic constituting the dry powder inhaler devices can have a strong impact on material loss during manufacturing of the product and upon actuation of the inhaler (Karner and Urbanetz, 2011).

Charged particles tend to adhere on the walls of the mixing vessel In the mixing process as

well as to the inhaler material during loading and discharging and thus influence the released dose (Karner and Urbanetz, 2011; Wong et al., 2015).

Selected excipients, known fort they lubricant properties (Pamunuwa et al., 2016), were screened for their ability to enhance flow (evaluated as emitted dose) and reduce particle cohesiveness of the spray-dried powders (measured as higher respirable dose). The putative charge-modifying agents could be either a neutral molecule, such as mannitol, or a positively charge excipients, such as L-lysine or stearylamine, chosen to neutralize the negative charge of hyaluronate. The effect of these surfactants on powder characteristics was assessed by adding them at fixed concentration (10% w/w) to the hyaluronate solution before spray drying.

The three hyaluronate:excipient solutions were spray dried and the powders characterized (see Table 2). The yield of the production process was in all the case higher than 49%. The hyaluronate content was around 80% w/v, in agreement with the consideration that a 10% of excipient was added and the formulation contained around 10% of water (as measured by TGA). The median volume diameter was equal to 5.41 and 2.78 μm when mannitol and lysine were added to the formulation, respectively (Figure 1A). These powders showed a broad particle size distribution with a span value of 1.16 and 2.44. The addition of these excipients improved the aerodynamic properties compared to the pure HYA powders, decreasing the powder entrapped in the device. When stearylamine was added as excipient, the emitted dose rose to 88% (Table 1; Figure 1B) and the HYA-stearylamine powder showed the narrowest particle size distribution (span 0.59, d_{v50} 2.59 μm). From the respirability data it was concluded that there was no direct correlation between the reduction of the hyaluronate charge in the solution for spray drying and the powder cohesiveness / aerodynamic behaviour.

Among the selected excipients, stearylamine provided the highest respirable fraction, and improved the microparticle de-aggregation during aerosolisation. The hyaluronate-stearylamine (HYA-SteAm) deposition in the fine fraction collector (FFC) reached 45% w/w (Figure 1B). This observation can be explained by the lubricant and surfactant properties of stearylamine. Stearylamine is a fatty primary amine acting as an emulsifying agent that will largely distribute at interfaces between air and water during droplet evaporation (Belotti et al., 2014; Parlati et al., 2009). This coating with a layer of lubricant results in a lower surface energy and lower powder cohesiveness.

3.2 Optimisation of sodium hyaluronate formulations for powder respirability

Based on the promising effects of stearylamine, other stearyl group-containing surfactants (cetostearyl alcohol and stearyl alcohol) were investigated to seek a further improvement of the powder performance. Three different hyaluronate:surfactant ratios (90:10, 95:5 and 99:1) were studied for each of these dispersion enhancers. The yield of the production process ranged between 44 and 66%, data not shown).

The *in vitro* respirability of these powders indicated that a reduction of the amount of surfactant in the spray-dried powders afforded a diminution of the emitted dose (Figure 2, Table 2). This behaviour was observed with all the three surfactants. The best result in terms of respirable fraction was achieved using 5% w/w of the excipient for all the stearyl surfactants. This concentration generated microparticles that de-aggregated readily, leading to a significantly higher respirable fraction compared to the formulations with 1% surfactant ($p < 0.001$ for all formulations). When the excipient was added at 1% w/w, the powders were not completely emitted from the device, similarly to the hyaluronate pure spray dried powder (HYA). When the stearyl surfactants were added at 10% w/w, the emitted dose was

acceptable (>75 % of the loaded dose) but the powder did not fully de-aggregate and it deposited in the induction port and in the coarse fraction collector (CFC) of the impactor (data not shown). The lack of linear relationship between surfactant content and *in vitro* respirability can be explained by assuming that at 10% w/w the surfactant concentration (0.92 mg/ml) is higher than its critical micelle concentration. The latter is reported in literature as 0.1 mg/ml in water (Wang, 2016). This concentration value is lower to the one employed in this study, however it has to be taken into account that the spray drying process was carried out in a water-ethanol mixture (70:30 v/v) in which the surfactant CMC is much higher due to the higher solubility of the surfactant itself. The micelle formation results in a partial internalization of the surfactant during the particle formation process (Parlati et al., 2009). Hence, these data suggest that in the spray drying conditions the optimal concentration of stearyl surfactant to afford a molecular coating at the particle hyaluronate surface is around 5% by weight. Although the RF value for the powder containing 10% cetostearyl alcohol was not statistically different from that of the powder containing 5% of the same surfactant ($p > 0.05$), a similar trend to that obtained with the other surfactants was observed.

SEM images (Figure 3) showed that the spray-dried powders containing the different stearyl surfactants exhibited marked differences in morphology. An effect of the surfactant concentration on the particle shape was observed as well. The excipient–free hyaluronate powder (containing only HYA) was constituted by particles having spherical shape with some concavities and a smooth surface. These samples presented some cohesive particle aggregates that account for the aerodynamic assessment observed with FSI (Table 1). Powders with stearylamine showed a different shape compared to spray-dried powder of pure HYA. An irregular wrinkled shape was produced which was more pronounced as the amount of the surfactant in the formulation increased. Powders containing HYA:cetostearyl alcohol in

different ratios had a roundish shape. The particles appeared to be sponge-like with number and size of the holes that were proportional to the surfactant concentration. Similar morphology was observed when stearyl alcohol was employed in the microparticle production.

To understand the reasons for the different morphology obtained for the amino-moiety containing particles compared to the alcoholic moiety containing particles investigations of the thermal properties of the particles as well as of the starting materials was carried out. Thermo-gravimetric analysis afforded a weight loss of 11.5-13.0% in the 30-140°C temperature range irrespective of the type of powder considered. The DSC traces of the starting materials and of the spray dried HYA powders are reported in Figure 4. Stearylamine presented a sharp peak at 57.2°C, followed by a second less pronounced endothermic event between 80 and 95°C. While the first peak represents the melting of the material, the second one is more difficult to interpret. Most likely it was due to an impurity as the labelled purity was 97% w/w. Stearyl alcohol and cetostearyl alcohol presented melting peaks at 60.3 and 55.9°C, respectively. In both cases the melting peak was preceded by a shoulder of even a first. Finally, sodium hyaluronate showed only a very broad endothermic event between 40 and 140°C which was attributed to moisture evaporation, in agreement with TGA data.

The powders constituted by HYA and stearylamine (Figure 4 panel b) did not present the stearylamine melting peak but only a small endothermic event. This latter tended to appear in advance (at lower temperature) as the concentration of the surfactant in the powder decreased. This means that the surfactant was present in the powder particles in amorphous form. On the contrary, the powders containing HYA, stearyl alcohol (Figure 4 panel c) and cetostearyl alcohol (Figure 4 panel d) presented an surfactant melting peak, whose dimension was proportional to the surfactant concentration. Interestingly, the DSC traces of these powders presented a second small endothermic event that was not observed in the traces of the pure

components. This event was around 75°C for the powders containing cetostearyl alcohol and 78°C for the powder containing stearyl alcohol. The peak temperature was consistently observed for the powders having the same components while its intensity was related to the surfactant concentration in the sense that the event could be observed only for the powders containing 5 and 10% surfactant. In agreement with data reported in the literature (Albèr et al., 2015; Průšová et al., 2013) the second peak was ascribed to the hyaluronan glass transition (Tg). In particular Albèr and co-workers (Albèr et al., 2015) reported a Tg of about 50°C for a polymer of slightly lower molecular weight (17 kDa) while the water moisture content was comparable to that observed in the present work; Prusova et al. (Průšová et al., 2013) provided DSC data affording a Tg ranging from 45.9 and 61.5°C in similar experimental conditions for a polymer of 800 kDa. Evidently, the polymer glass transition could be observed only in the presence of a certain amount of surfactant.

The thermal analysis provides a possible explanation for the different particle morphologies between the powders containing stearylamine and those containing either stearyl alcohol or cetostearyl alcohol, shown in Figure 3. Particles containing stearylamine were amorphous in nature in particular with respect to the surfactant that constituted the particle shell. Being amorphous this shell was likely quite plastic allowing for the particle collapse during the solidification, thus leading to the observed corrugated morphology. This behaviour has been observed for peptides particles as spray-dried insulin that showed a collapsed and wrinkled surface morphology (Balducci et al., 2013). On the other hand, the melting peak of the surfactants in the stearyl alcohol- and cetostearyl alcohol-containing particles, suggest that these surfactant crystallized between the spray drying inlet and outlet temperatures (90-50°C), i.e. in the drying chamber of the spray drier when the boiling and solvent evaporation occurred. The formation of a crystalline shell provided a rigid structure pierced by the evaporating solvent bubbles, thus affording the porous sponge-like structure. A similar

particle morphology and evaporation mechanism was observed during the formation of BDP particles from solution-based pMDI. It is reported that the maximization of evaporation rate leads to a rapid escape of the boiling liquid from within the particle giving rise to the formation of porous structures with irregular surface geometry (Buttini et al., 2014) (Lewis et al., 2014).

The morphology enabled the aerodynamic behavior observed to be rationalized. It is reported that the shape and presence of the holes on the particle surface (porous particles) reduced the effective surface area available for particles contact and cohesion, decrease the density, resulting in improved flowability (higher emitted dose) and de-aggregation (higher respirable dose). It was concluded that the HYA:surfactant ratio 95:5 afforded the best aerodynamic performance. Thus, spray-dried powders with 5% stearyl surfactant composition were more fully characterized with respect to their aerodynamic behavior using the ACI. The data generated with the ACI confirmed the results obtained with the FSI demonstrating that the presence of a surfactant improve the aerodynamic performance of HA powder; with HA: stearylamine (95:5) having the best aerodynamic performance among all the dry powder formulations tested (Table 3; Figure -5).

3.3 Biocompatibility of hyaluronate powders with lung cells

Biocompatibility with human alveolar epithelial cell *in vitro* was evaluated for the powder formulations and their individual constituents using the human alveolar epithelial cell line, A549, which is widely used in inhalation toxicology (Zavala et al., 2016). This cell line was selected as it is representative of the mucosa at the intended site of action of the HYA particles in the peripheral lung. To date, there are no toxicological data available for the materials employed in this study when delivered by inhalation. The powder formulations with

the highest surfactant concentration (10%) were screened for biocompatibility as they possessed the highest potential for toxicity. The data show that HYA alone did not induce overt toxicity after 24 h of exposure (Figure 6A). Although a systematic lowering of the MTT assay readout was noted, cell viability remained above 50% of the untreated control at all concentrations tested. For the formulations containing stearyl- surfactants dose dependent cytotoxicity was observed (Figure 6B). The effect of individually applied stearyl- surfactants on A549 cells ranked similarly to the effect of the formulations containing the same surfactants, leading us to conclude that loss of cell viability after exposure to the powders was due to the presence of the surfactant component, although somewhat mitigated by co-administration with HYA in a 10:90 ratio.

Stearylamine was the most potent of the surfactants in reducing cell viability, LC_{50} = 1.81 µg/mL, whereas the least toxic surfactant stearyl alcohol was ten times less potent in terms of LC_{50} (Table 4). The data show that the relative biocompatibility of formulations was HYA-stearyl alcohol > HYA-cetostearyl alcohol > HYA-stearylamine, with the surfactants alone possessing the same ranking, albeit with lower LC_{50}.

The LC_{50} value of excipients approved for inhalation measured using the MTT assay with respiratory epithelial cells is typically > 500 mM, with surfactants being more potent, e.g. for polysorbates the LC_{50} value was 0.9-8.0 mM (Scherließ, 2011). For the HYA powder containing 5% of stearyl alcohol, the fine particle dose was 1.98 mg from 5 mg of powder loaded in a capsule (Table 3). Hence, the stearyl alcohol that potentially reaches the lung can be estimated to be approximately 0.1 mg (5% of the 1.98 mg respirable dose). Although not taking into account local concentration gradients during particle dissolution, solubilisation of this dose in the available volume of lung lining fluid (20 mL based on estimates of 10-30 mL in the conducting airways and 7-20 mL in the alveolar region (Hastedt et al., 2016), would

provide a surfactant concentration of around 5 µg/ml, namely 0.018 mM, which compares very favorably to an LC_{50} = 24.7 mg/mL *in vitro*. Therefore, this data represent a preliminary encouraging step toward the gain the FDA approval for this new excipients . Considering the need to provide the maximum safety margin, and to balance the findings for biocompatibility versus aerosol performance, HYA powders containing 5% stearyl alcohol were determined to be the most promising for further studies.

Conclusions

A method for engineering respirable hyaluronate powders has been developed using a spray-drying process. Particles with a shape and morphology favourable for aerosolization were obtained by manipulating the type and concentration of surfactants in the formulation. Based on *in vitro* evaluations of respirability and biocompatibility, a lead formulation containing 5% stearyl alcohol was identified as a highly promising prototype for translating the proposed benefits of inhaled hyaluronate into commercially viable, safe and clinically effective hyaluronate products.

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Figure Legends

Figure 1. Particle size distribution of spray-dried powders of sodium hyaluronate ± excipients (10%). A. Laser diffraction of hyaluronate powder (HYA, blue) or powders of sodium hyaluronate containing lysine (HYA-Lysine, green), mannitol (HYA-Mannitol, red) or stearylamine (HYA-stearylamine, black). B. The deposition of the powders in the fast screening impactor, Device = RS01 capsule powder inhaler, IP = induction port, CFC = coarse fraction collector, FFC = fine fraction collector (particles < 5 µm; respirable fraction). Data represent mean ± standard deviation, n=3.

Figure 2. Respirable fraction measured by fast screening impactor of powders spray dried from hyaluronate:stearylamine (HYA-StAm), hyaluronate:cetostearyl alcohol (HYA-CetSteAlc) and hyaluronate:stearyl alcohol (HYA-SteAlc) in ratio of 99:1, 95:5, 90:10. Data represent mean ± standard deviation, n=3.

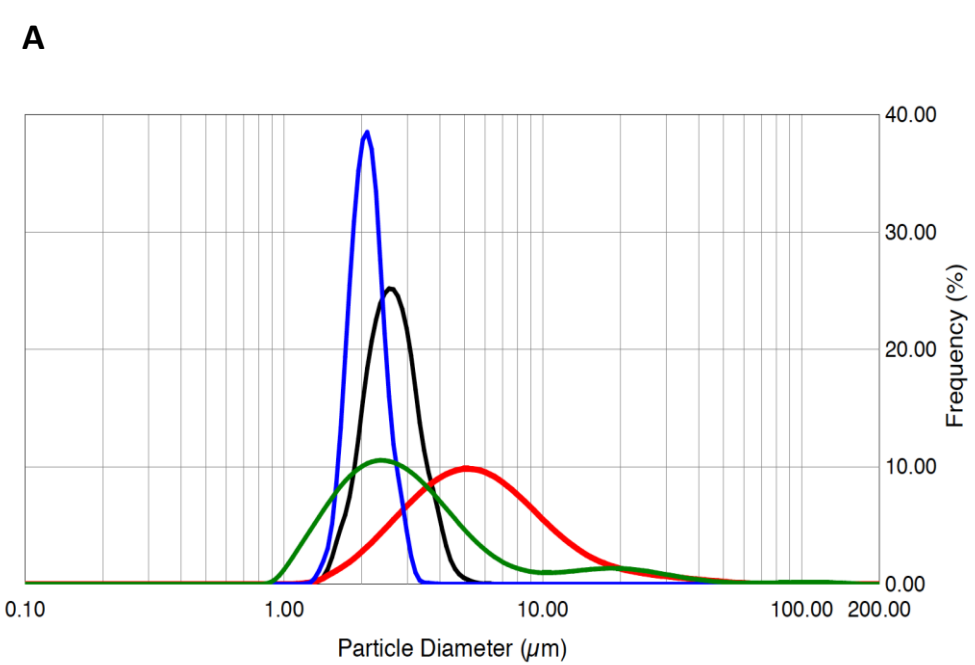
Figure 3. Scanning electron microscopy images of hyaluronate spray-dried powder (surfactant-free) and hyaluronate:stearyl surfactants in ratio of 99:1, 95:5, 90:10. Picture magnifications are in the range 30-50000 x.

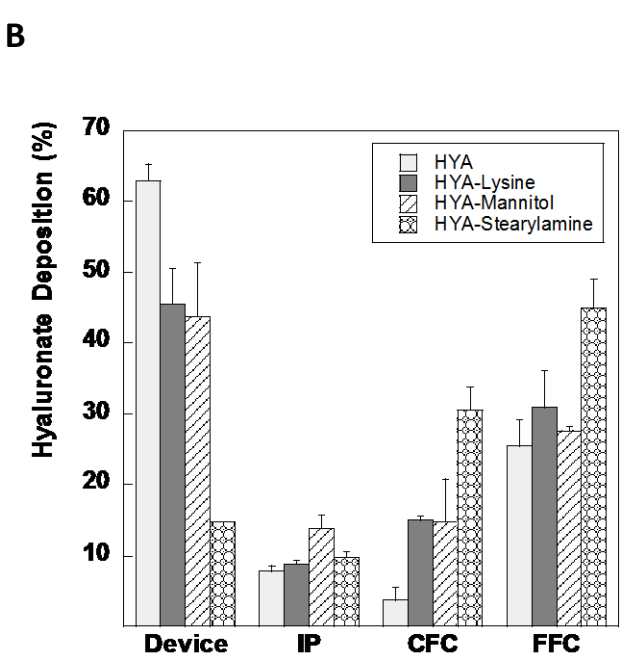
Figure 4. DSC thermograms of hyaluronate and excipient raw materials (panel a) and spray dried powders containing hyaluronate and surfactants in different ratio: hyaluronate:stearylamine (HYA-StAm, panel b), hyaluronate:stearyl alcohol (HYA-SteAlc, panel c) and hyaluronate:cetostearyl alcohol (HYA-CetSteAlc, panel d).

Figure 5. Distribution on the stages of an Andersen cascade impactor of the hyaluronate:surfactant 95:5 spray-dried powders: HYA:stearylamine (HYA-StAm-5), HYA:stearyl alcohol (HYA-SteAlc-5) and HYA:cetostearyl alcohol (HYA-CetSteAlc-5). D&C = device and capsule, IP = induction port, S1-6 =stages 1 to 6, F = filter. Powders were aerosolised using an RS01 capsule powder inhaler device (loaded dose 5 mg). Data represent mean ± standard deviation, n=3.

Figure 6. Biocompatibility of sodium hyaluronate and surfactants alone and in the spray dried powders with A549 alveolar epithelial cells evaluated using an MTT assay for cell viability. A. Stearyl surfactants or hyaluronate (HYA) applied as single agents. B. hyaluronate:surfactant 90:10 spray-dried powders. Powders were applied in solution to A549 cells for 24 h, data represent mean ± SD (n=18; three independent experiments with 6 replicates at each concentration).

Figures





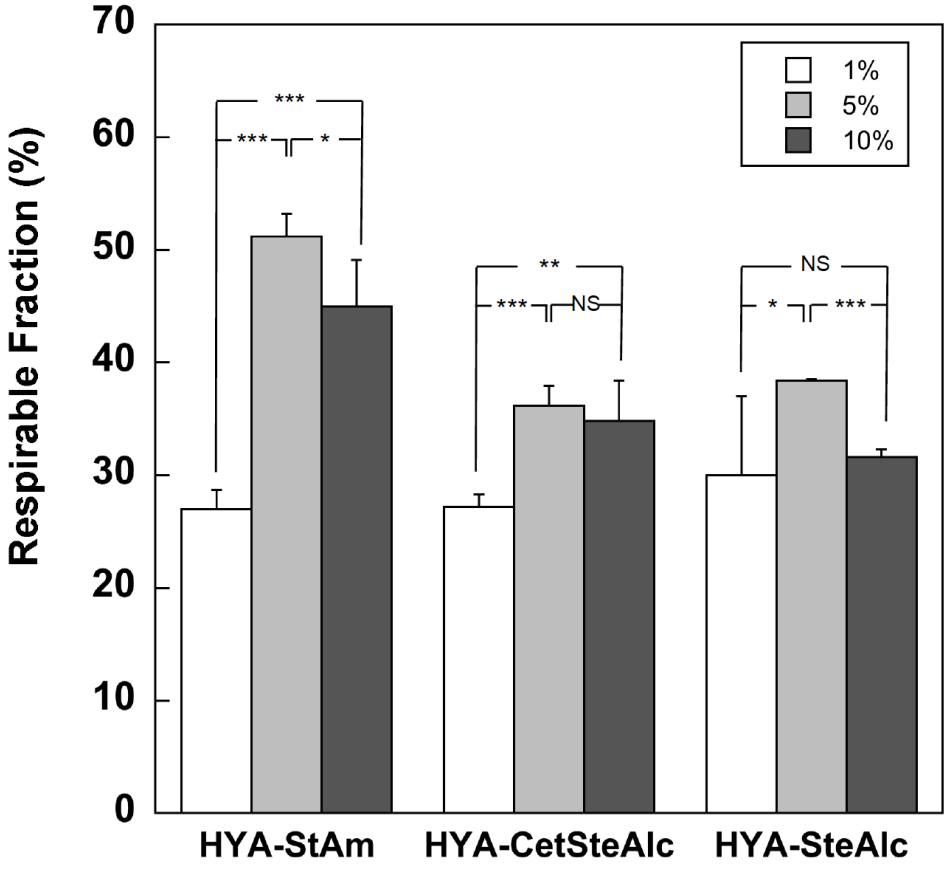
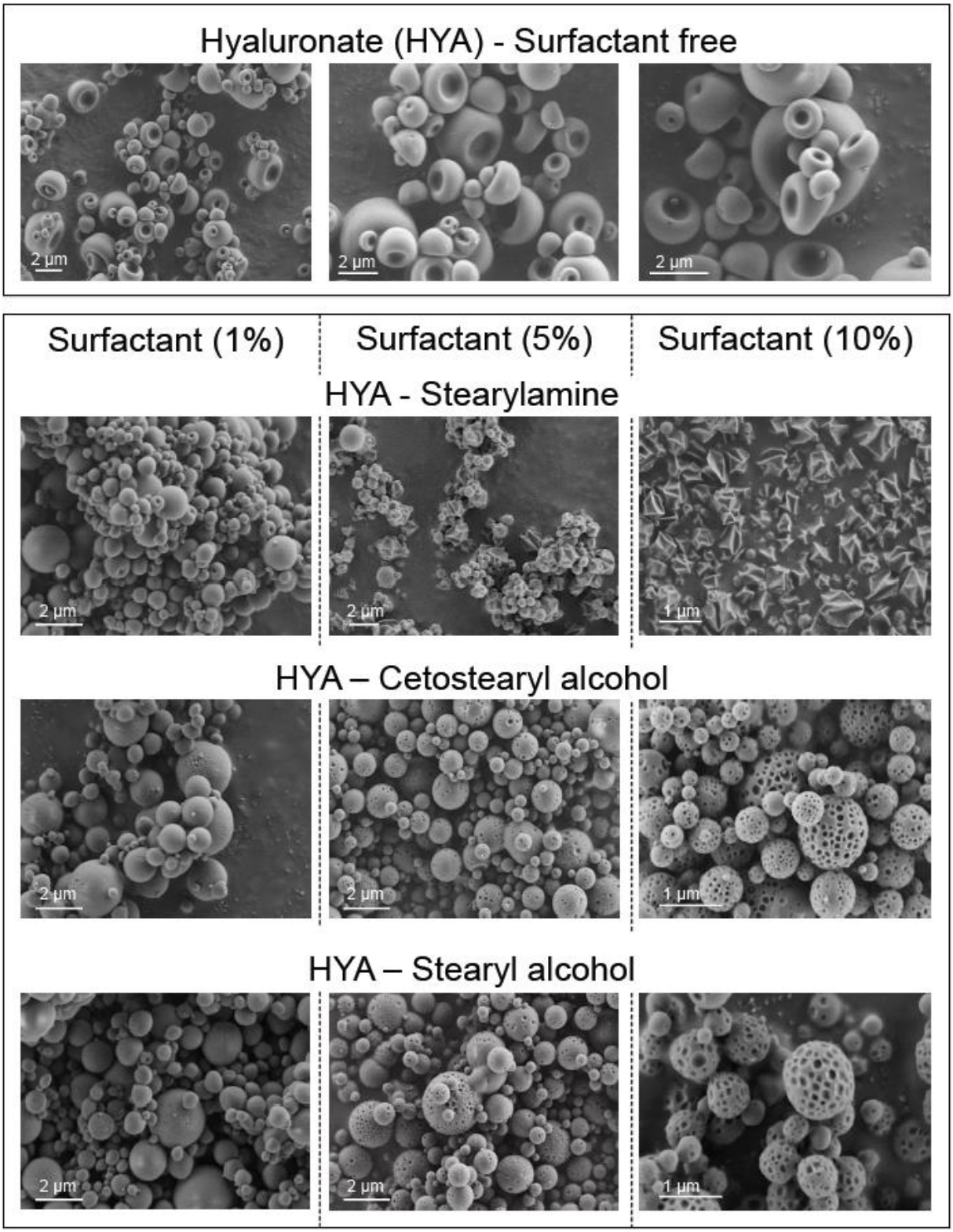


Figure 2



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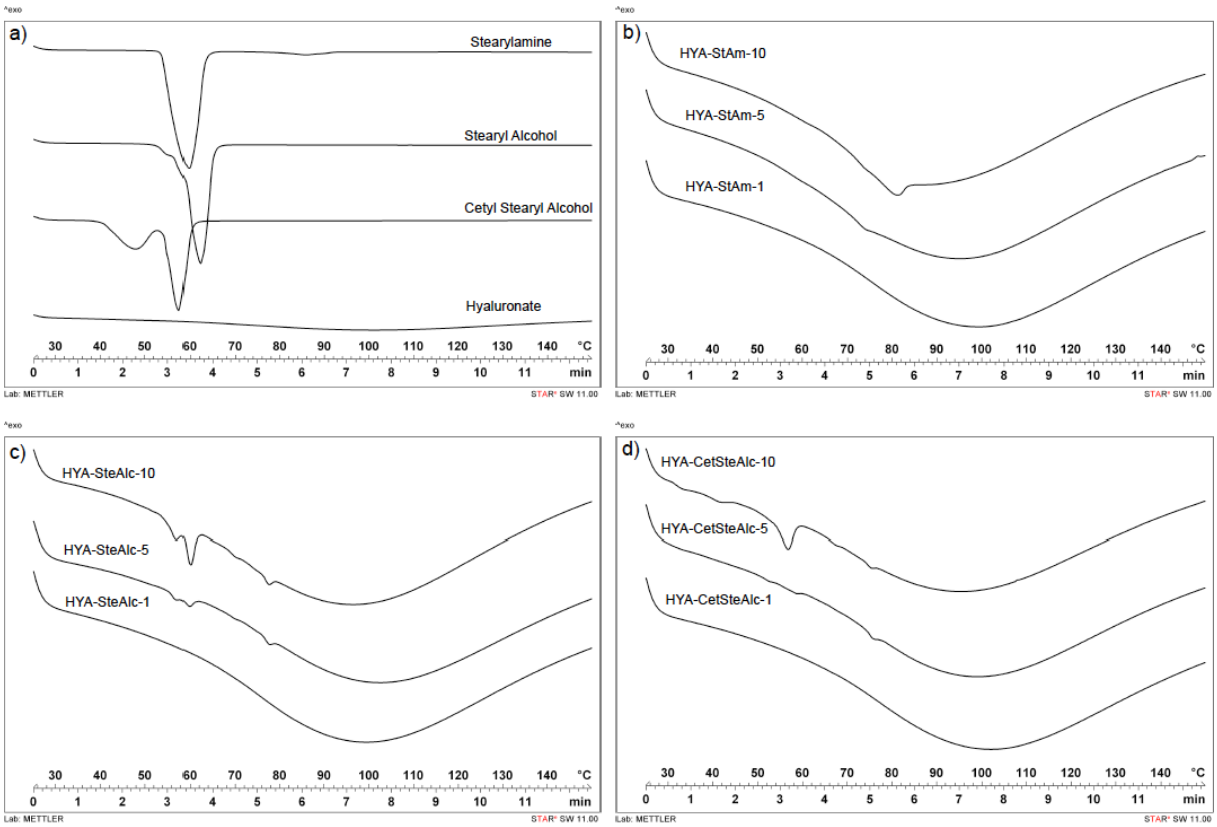


Figure 4

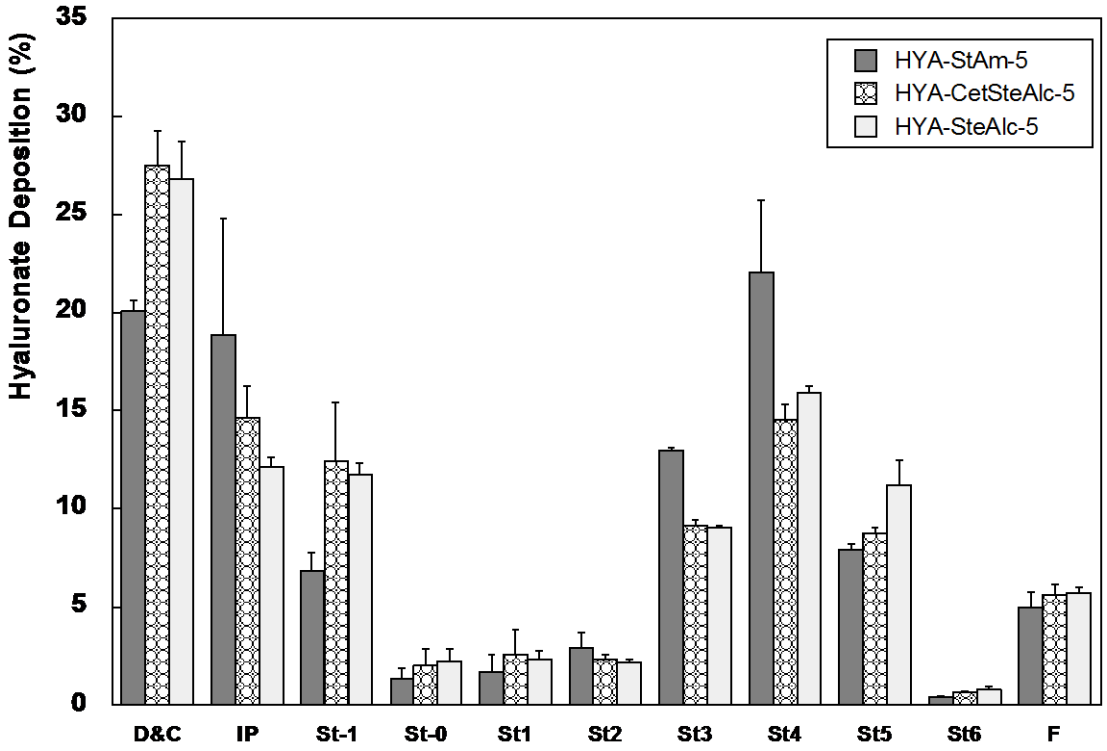
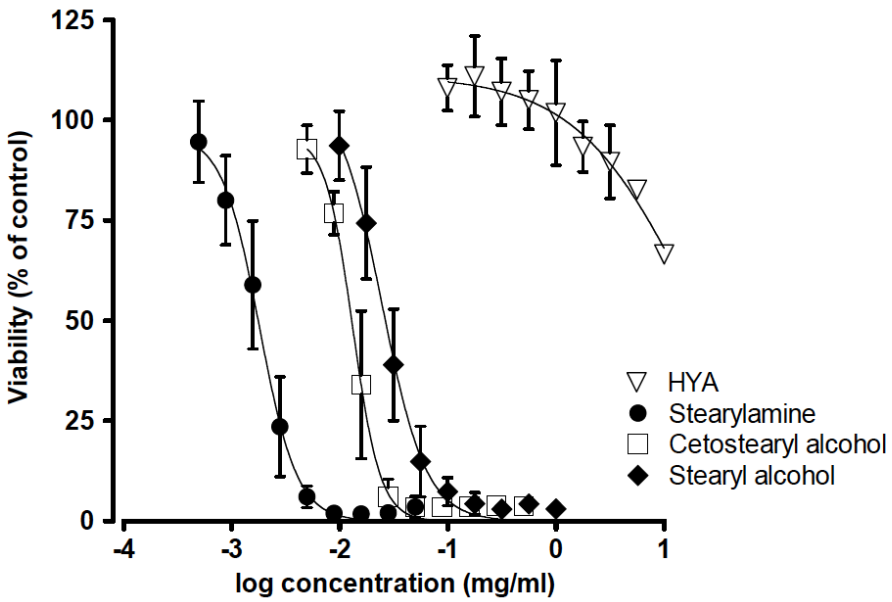


Figure 5

A



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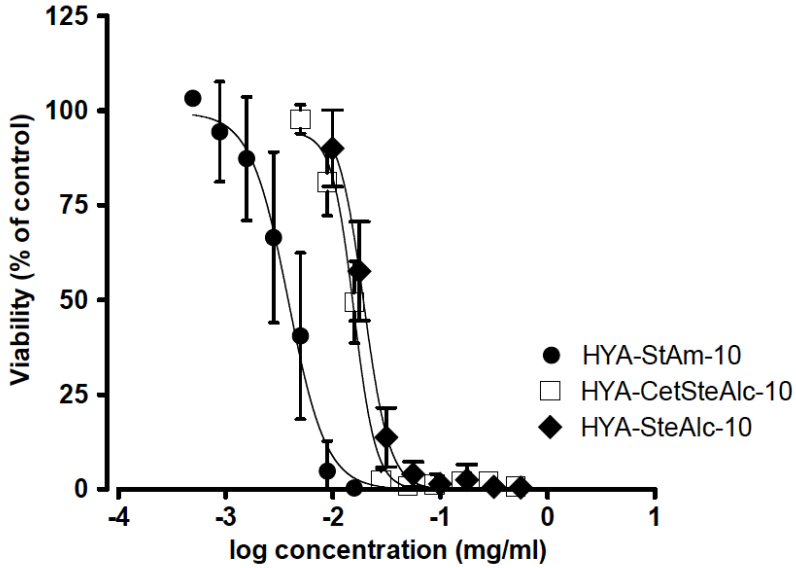

Figure 6

Tables

Table 1. Hyaluronate formulations screened to identify excipients that enhance spray drying yield, emitted dose and respirable fraction. Data represent mean ± standard deviation, n=3.

	Feed solution to spray drying					**Powder characterization**		
Hyaluronate powder formulation	**Surfactant type**	**Surfactant ratio to hyaluronate (% w/w)**	**Solute content of feed suspension (% w/v)**	**Surfactant concentration (mg/ml)**	**Production yield (%)**	**HYA content (%)**	**Emitted dose (%)**	**Respirable fraction (%)**
Hyaluronate (HYA)	-	-	0.83	-	67.9 ± 2.1	91.1 ± 0.5	38.6 ± 0.10	25.4 ± 3.7
HYA-Mannitol	Mannitol	10	0.92	0.92	65.0 ± 3.2	80.6 ± 0.8	67.4 ± 0.11	27.6 ± 0.6
HYA-Lysine	Lysine	10	0.92	0.92	76.7 ± 1.4	79.8 ± 0.8	58.7 ± 0.05	30.9 ± 5.2
HYA-Stearylamine	Stearylamine	10	0.92	0.92	49.0 ± 2.4	78.9 ± 2.0	88.1 ± 0.01	45.0 ± 4.1

Table 2. Optimisation of hyaluronate:stearyl surfactant ratio by evaluation of spray dry production yield and inhalation performance. Composition of the feed solution to spray drying is reported A loaded dose of 5 mg powder in a capsule inhaler device RS01 was evaluated using the fast screening impactor at 60 L/min to measure emitted dose and respirable fraction of the spray-dried hyaluronate powders. Data represent mean ± standard deviation, n=3.

	Feed solution to spray drying				**Powder characterization**		
Hyaluronate powder formulation	**Stearyl surfactant**	**Surfactant ratio to hyaluronate (% w/w)**	**Solute content (% w/v)**	**Surfactant concentration (mg/ml)**	**Hyaluronate content (%)**	**Emitted dose (%)**	**Respirable fraction (%)**
HYA-StAm-1	Stearylamine	1	0.84	0.084	88.1 ± 0.9	58.2 ± 0.20	27.0 ± 1.7
HYA-StAm-5		5	0.88	0.44	83.2 ± 0.4	80.1 ± 0.29	51.2 ± 2.0
HYA-StAm-10		10	0.92	0.92	78.3 ± 1.9	88.1 ± 0.01	45.0 ± 4.1
HYA-CetSteAlc-1	Cetostearyl alcohol	1	0.84	0.084	90.1 ± 1.2	71.8 ± 0.08	27.2 ± 1.1
HYA-CetSteAlc-5		5	0.88	0.44	86.4 ± 1.7	76.1 ± 0.14	36.2 ± 1.7
HYA-CetSteAlc-10		10	0.92	0.92	79.2 ± 0.7	91.5 ± 0.01	34.8 ± 3.6
HYA-SteAlc-1	Stearyl alcohol	1	0.84	0.084	89.4 ± 1.2	69.1 ± 0.07	30.0 ± 7.0
HYA-SteAlc-5		5	0.88	0.44	85.4 ± 1.5	75.9 ± 0.01	38.4 ± 0.1
HYA-SteAlc-10		10	0.92	0.92	79.6 ± 1.1	90.4 ± 0.05	31.6 ± 0.7

Table 3. Characterisation of the aerosol properties of the powders with the excipient ratio resulting in the highest respirable fraction (hyaluronate:stearyl surfactant ratio 95:5). Powders (5 mg) were aerosolised using an RS01 capsule powder inhaler device and deposition was measured in an Andersen impactor operated at 60 L/min. ED = emitted dose, MMAD = mass median aerodynamic diameter, FPD = fine particle dose, FPF = fine particle dose, RF = respirable fraction. Data represent mean ± standard deviation, n=3.

Hyaluronate powder formulation	**ED (%)**	**MMAD (µm)**	**FPD (mg)**	**FPF (%)**	**RF (%)**
HYA-StAm-5	79.9 ± 0.5	1.96 ± 0.12	2.20 ± 0.21	66.3 ± 5.6	49.4 ± 3.5
HYA-CetSteAlc-5	72.5 ± 1.7	2.28 ± 0.36	1.80 ± 0.08	59.0 ± 2.5	38.6 ± 2.1
HYA-SteAlc-5	73.2 ± 1.9	2.03 ± 0.13	1.98 ± 0.05	64.0 ± 0.6	43.8 ± 0.9

- FPF = FPD/amount collected in the impactor
- RF= FPD/loaded dose

Table 4. Comparison of LC_{50} (50% of the lethal concentration) for stearyl surfactants alone or in combination (10:90 ratio) with sodium hyaluronate. The agents were applied to human alveolar epithelial cells in solution for 24 h. The data represent the mean and the 95% confidence interval (CI) of n=3 individual experiments.

Stearyl surfactant / powder formulation compositions	**LC_{50} (95% CI) (µg/ml of surfactant)**
HYA-StAm-10	3.85 (3.09-4.79)
Stearylamine	1.81 (1.51-2.18)
HYA-CetSteAlc-10	15.59 (14.42-16.86)
Cetostearyl alcohol	13.18 (11.53-15.06)
HYA-SteAlc-10	19.42 (17.17-21.97)
Stearyl alcohol	24.65 (18.37-33.07)